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A survey of human papillomavirus 16 antibodies in patients with epithelial cancers

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Human papillomavirus (HPV), particularly HPV 16, is linked to the development of cervical cancer. However, the role of HPV 16 in a number of extra-cervical epithelial tumours is controversial. To assess exposure to HPV 16 in patients with different cancers, we conducted a large serosurvey of 905 patients with 21 types of tumours and measured IgG to HPV 16 virus-like particles (VLPs) using a well characterized enzyme linked immunosorbent assay (ELISA). Patients with cervical cancer were considered 'positive controls', as about half were expected to be specifically HPV 16-related. A non-cancer study group consisting of 48 patients with endocrine disorders (*eg* diabetes) was also tested. HPV 16 antibody prevalence was highest in patients with cancers of the cervix (52% ± 7%), vulva (27% ± 9%), vagina (27% ± 13%) and penis (63% ± 16%). Seroprevalence was much lower in the non-cancer group (4% ± 3%) and all other cancer patients: uterus (9% ± 4%); ovary (4% ± 3%); testis (5% ± 4%); prostate (6% ± 4%); squamous carcinoma (6% ± 3%) and adenocarcinoma (4% ± 3%) of the lung; rectum (2% ± 2%); pancreas (8% ± 4%); colon (2% ± 2%); stomach (0%); oesophagus (8% ± 4%); buccal cavity (12% ± 5%); breast (10% ± 4%); non-melanomatous (9% ± 6%) and melanomatous (6% ± 3%) skin tumours; bladder (8% ± 4%); and kidney (2% ± 2%). The results confirm the strong relation of HPV with several lower anogenital tract tumours, but, at least in this population, fail to identify additional epithelial tumours associated with high seroprevalence of HPV 16 VLP antibodies.

Key words: Antibodies, cancer, carcinoma, HPV 16, human papillomavirus, virus-like-particles, VLP.

Introduction

Human papillomavirus (HPV) infection is linked to the development of cancer of the cervix and its precursors (Lorincz *et al*, 1992; Schiffman *et al*, 1993). Almost all cervical cancer specimens contain HPV DNA (Bosch *et al*, 1995). In the USA and Europe more than half of these tumours contain a particular HPV type, HPV 16 (Bosch *et al*, 1995). Antibodies to HPV 16 virus-like particles (VLPs), consisting of the viral proteins that form the virus capsid, are strongly associated with detection of HPV 16 DNA in cervical epithelium (*ie* current HPV 16 infection) and also with cervical neoplasia (Kirnbauer *et al*, 1994; Strickler *et al*, 1997a). Recent reports confirm that this antibody is at least partly specific for HPV 16 (Wang *et al*, 1997) and that seroassay results can be generalized between laboratories (Strickler *et al*, 1997b). Notably, the highest HPV 16 VLP seroprevalence rates are in individuals who have HPV 16

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infection-related neoplastic lesions that persist (de Gruijl *et al*, 1997), an important factor in the development of frank carcinoma (Chua and Hjerpe, 1996). Moreover, serological studies using prospectively collected blood specimens have been important in confirming the temporal relationship of HPV 16 infection with the later development of cervical cancer (Shah *et al*, 1997).

The link between HPV 16 and cervical carcinoma has led investigators to search for additional HPV 16-cancer associations, based primarily on the search for HPV DNA in tissue specimens. HPV 16 is sexually transmitted and highly tropic for epithelial cells (IARC, 1995). Consistent with this, epithelial cancers of the anus (Holm *et al*, 1994; Palefsky, 1994), penis (Maden *et al*, 1993; Malek *et al*, 1993; Gregoire *et al*, 1995), vulva (Schneider *et al*, 1991; Trimble *et al*, 1996), and vagina have each been reported to commonly contain HPV 16 DNA (Ikenberg *et al*, 1990; Schneider *et al*, 1991) and, less often, the DNA of other cervical cancer-related HPV types (IARC, 1995). The literature is less clear regarding the presence of HPV DNA in other anogenital tumours, including cancers of the prostate, uterus, ovaries and testes (Sinclair *et al*, 1993; Fujita *et al*, 1995; IARC, 1995; Trottier *et al*, 1995; Czerwenka *et al*, 1996; Rajpert-De Meyts *et al*, 1996; Strickler *et al*, 1998).

HPV types that infect anogenital epithelium can also infect the upper aerodigestive tract, as in laryngeal papillomatosis (generally caused by HPV 6/11), a benign tumour of children (IARC, 1995). Several recent studies have reported detection of HPV DNA, most commonly HPV 16, in epithelial cancers of the aerodigestive tract (Kulski *et al*, 1990; Lewensohn-Fuchs *et al*, 1994; IARC, 1995; Franceschi *et al*, 1996) including cancers of the buccal cavity (Ostwald *et al*, 1994; Steenbergen *et al*, 1995; Nielson *et al*, 1996), pharynx/larynx (Kulski *et al*, 1990; Lewensohn-Fuchs *et al*, 1994), tonsil (Lewensohn-Fuchs *et al*, 1994), oesophagus (Kulski *et al*, 1990; Chen *et al*, 1994), and even lung (Kulski *et al*, 1990; Quingquan *et al*, 1995). Other epithelial tumours throughout the body have also been occasionally reported to contain anogenital HPV DNA, again predominantly HPV 16, including cancers of the bladder (Agliano *et al*, 1994; Lopez-Beltran and Muñoz, 1995), kidney (Furihata *et al*, 1995), breast (Di Lonardo *et al*, 1992), and colon (Cheng *et al*, 1995). Some skin cancers may also contain anogenital HPV DNA (Stancu *et al*, 1996). However, as a whole, there have been relatively few studies of HPV DNA in non-anogenital tumours. The studies conducted to date have generally been small and the findings have been inconsistent, sometimes negative (Kulski *et al*, 1990; Bratthauer *et al*, 1992; Shah *et al*, 1992; Szabo *et al*, 1994; IARC, 1995; Smits *et al*, 1995). Furthermore, HPV DNA detection methods (*eg* polymerase chain reactions) are prone to false-positive results (Sinclair *et al*, 1993; IARC, 1995; Franceschi *et al*, 1996). Thus, the relation of anogenital HPV infection with any non-anogenital cancers is uncertain.

To study HPV 16 exposure in patients with extracervical epithelial cancers, we conducted a large serosurvey of HPV 16 VLP antibodies in patients with 21 types of tumours that have varying degrees of evidence linking them to HPV infection. HPV 16 VLP serology was particularly useful for this purpose. In addition to being a valid measure of current HPV 16 infection, independent of DNA testing, serum antibodies persist for a variable period of time and may reflect earlier infections with HPV 16 (Carter *et al*, 1996). Therefore, even if HPV 16 were to play a role during an early phase in tumorigenesis of a particular cancer but was not maintained in cancer cells (*ie* a so called 'hit and run' effect) an association might still be detectable using serology. We note that cross-sectional HPV antibody data, by themselves, may provide limited information on the aetiology of a cancer. However, a high prevalence of HPV 16 antibodies would be an indication for a comprehensive virological and epidemiological study.

Material and methods

Specimens

The National Cancer Institute (NCI) Immunodiagnosis Serum Bank, established by NCI through contact with the Mayo Clinic, provided sera from hospitalized patients in Minnesota with any of 21 selected cancers (Table 1). The cancer types were chosen because HPV 16 had been reported in tumour specimens in previous studies, or the cancers had not been studied (*eg* stomach, pancreas). The specimens tested were collected by the serum bank between 1975 and 1991, as part of a broader project to obtain blood specimens from in-patients diagnosed with 85 different types of cancers, benign tumours and non-tumour related conditions. The goal was to maintain specimens from 50 different patient with each tumour diagnosis. All sera were stored at –70°C until tested (Di Magno *et al*, 1989).

Patients with cervical cancer were considered 'positive controls', as the relation of HPV 16 with cervical cancer is well defined; about half of cervical cancer cases were expected to be specifically HPV 16-related

(Lorincz *et al*, 1992; Bosch *et al*, 1995). There was one non-cancer control group of 48 patients with endocrine disorders (*eg* diabetes). However, this investigation was mainly intended as a descriptive serosurvey of cancer patients (see statistical analysis).

Seroassay

Laboratory personnel were masked to patients' diagnoses, and specimens were randomly ordered to avoid batch effects. HPV-16 VLPs were prepared from sf9 insect cells infected with a recombinant baculovirus expressing the L1 and L2 proteins of HPV-16, and purified by the method of Kirnbauer *et al* (1993). In brief, the purified VLP preparations were coated overnight at 4°C on to wells of a 96-well polystyrene microtitre plate (Nunc-Immuno Polysorp™, Fisher Scientific, Pittsburgh, PA) at a total protein concentration of 2 μg/ml and a volume of 100 μl in phosphate buffered saline (PBS). After washing the plate five times with PBS-0.05% Tween 20 (PBS-T), 100 μl of test serum diluted 1:10 in PBS-0.5% non-fat milk was added to each well. The plate was incubated for 2 h at 37°C, then washed five times with PBS-T. VLP-reactive antibodies were detected with horseradish peroxidase-conjugated recombinant Protein G (Zymed Laboratories, San Francisco, CA) diluted 1:20,000 in PBS-T. After a 1 h incubation at 37°C with the conjugate, freshly prepared ABTS and hydrogen peroxide solution (Kirkegaard & Perry, Gaithersburg, MD) were added to the wells and the plate was incubated for 30 min at room temperature. The optical density (OD) was read in a microtitre plate reader (Molecular Devices Corp, Menlo Park, CA) at 405 nm. One human serum sample previously found to be reactive was included on each plate as a positive control. The negative control on each plate was a serum sample previously found not to be reactive. The OD values for control specimens were required to be within a limited range or the entire plate was considered inadequate and re-tested. For additional quality control, each serum specimen was tested in duplicate. Samples were tested again if the OD values in the duplicate wells were widely disparate (*ie* a ratio >2.0 or <0.5) and either result was at least 50% of the positive cut-off value (to minimize re-testing owing to small variations among low values). Re-testing was conducted in 9% of samples. The ELISA value analysed was then the mean of the OD results for a given specimen measured on adequately tested plates. A high level of agreement between the assay results in this laboratory and others has been demonstrated previously (Strickler *et al*, 1997b).

Statistical analysis

Age and sex, as well as treatment history, were summarized separately for each cancer. Seroprevalence rates were estimated from ELISA OD values categorized as positive, negative or indeterminate based on cut-offs determined in previous studies (Viscidi *et al*, 1997) and standard errors were used to measure the precision of these estimates.

We chose, *a priori*, a descriptive serosurvey approach to the study of antibody prevalence rates because the cross-sectional examination of so many types of cancer created a large number of potential comparisons, making formal statistical tests difficult to interpret. Similarly, it would have been improper to conduct a series of 21 separate case-control comparisons to examine which tumour patients were statistically more likely than controls to have antibodies. In any event, assuming that some cancers were truly HPV-16 associated and others were not, two easily discernible and divergent seroprevalence distributions (high *vs* low) were expected. All data are presented in full.

Results

Serum specimens from 48 non-cancer controls and 905 patient with 21 different types of cancer were tested for antibodies to HPV 16. Cancer type, sample size, age, sex and history of treatment, are summarized in Table 1. For most cancers studied there were about 50 subjects tested. However, for cancers of the penis ($n = 8$), vagina ($n = 11$), non-melanomatous skin cancer ($n = 23$), vulva ($n = 26$), and testis ($n = 39$) there were smaller sample sizes. Mean age and the percentage of males and females varied in predictable ways, consistent with the epidemiology of the various cancers. For example, patients with testicular cancer were generally younger men (mean age, 32 years), and patients with ovarian cancer were generally older women (mean age, 62 years). Most patients (63%) had received no treatments prior to donating the blood tested in this study, and an additional 12% were bled within 1 month of initial treatment. Only 10% of blood specimens were obtained more than 1 year after initial treatment.

Non-cancer controls and genital cancer cases

HPV 16 VLP seroprevalence estimates and standard errors are shown according to detailed patient diagnoses in Table 1 and summarized in a simplified form in Figure 1. Seroprevalence was low among non-cancer controls. In contrast, the greatest antibody prevalence was among patients with lower genital

Table 1. Characteristics of patients and HPV 16 VLP antibody ELISA results

Cancer type	*n*	Positive		Mean age (years)	Percent female	Percent with prior treatment†		
		Number	Percent			None	< 1 year	> 1 year
Controls and non-genital cancers								
Controls (endocrine disease)	48	2	4 (± 3)‡	51	52	NA	NA	NA
Kidney	50	1	2 (± 2)	62	38	82	12	6
renal cell carcinoma	44	1	2					
other	6	0	0					
Bladder (transitional cell)§	49	4	8 (± 4)	69	24	29	35	37
Melanoma	50	3	6 (± 3)	56	50	21	41	38
Non-melanoma skin cancers	23	2	9 (± 6)	64	39	35	43	22
basal cell	9	0	0					
squamous cell	8	0	0					
verrucous carcinoma	1	1	100					
bowens disease	1	1	100					
other	4	0	0					
Breast	49	5	10 (± 4)	55	100	74	26	0
intraductal carcinoma	34	3	9					
lobular carcinoma	4	2	50					
adenocarcinoma	2	0	0					
other	9	0	0					
Aerodigestive tumours								
Buccal	50	6	12 (± 5)	61	36	62	4	34
squamous cell	38	5	13					
mucoepidermoid	4	1	25					
other	8	0	0					
Oesophagus	50	4	8 (±4)	62	22	77	8	15
adenocarcinoma	27	3	11					
squamous cell	19	1	5					
other	4	0	0					
Stomach	49	0	0 (± 0)	63	10	82	4	14
adenocarcinoma¶	34	0	0					
signet ring cell	13	0	0					
other	2	0	0					
Colon	50	1	2 (± 2)	68	38	63	23	15
adenocarcinoma¶	40	1	3					
pseudomyxoma	10	0	0					
Pancreas	49	4	8 (± 4)	61	37	78	20	2
adenocarcinoma	20	1	5					
intraductal carcinoma	18	2	11					
other	11	1	9					
Rectum (adenocarcinoma)¶	51	1	2 (± 2)	62	31	44	30	26
Lung adenocarcinoma	51	2	4 (± 3)	64	35	90	8	2
squamous carcinoma	49	3	6 (± 3)	66	10	96	4	0
Genital cancers								
Prostate	47	3	6 (± 4)	69	0	61	17	22
acinar cell	30	1	3					
adenocarcinoma	16	2	13					
other	1	0	0					
Testis	39	2	5 (± 4)	32	0	39	26	34
embryonal carcinoma	14	1	7					

Table 1. Continued

Cancer type	*n*	Positive		Mean age (years)	Percent female	Percent with prior treatment†		
		Number	Percent			None	< 1 year	> 1 year
teratoma	12	0	0					
seminoma	10	1	10					
other	3	0	0					
Penis (squamous carcinoma)	8	5	63 (± 16)	69	0	50	38	13
Ovary	50	2	4 (± 3)	63	100	24	28	48
adenocarcinoma	16	1	6					
cystadenoma	27	1	4					
other	7	0	0					
Uterus	54	5	9 (± 4)	62	100	67	11	22
adenocarcinoma	39	4	10					
other	15	1	7					
Vagina	11	3	27 (± 13)	59	100	50	0	50
squamous carcinoma	8	3	38					
other	3	0	0					
Vulva (squamous carcinoma)‖	26	7	27 (± 9)	66	100	59	23	18
Cervix	50	26	52 (± 7)	45	100	66	8	26
squamous carcinoma	39	23	59					
adenocarcinoma	7	1	14					
Other/not specified	4	2	50					

†Whether/when patients were treated prior to date blood donated. Some values do not add up to 100 of rounding; NA = not applicable
‡Seroprevalence (± standard error)
§One tumour was an adenocarcinoma rather than a transitional cell cancer.
¶One cancer was a carinoid tumour.
‖One tumour was a melanoma rather than a squamous carcinoma.

tract tumours, particularly patients with cancers of the penis and cervix. Seroprevalence was intermediate in cancers of the vulva and vagina. By comparison, antibody prevalence rates were markedly lower among other genital tumours, including prostate, testis, ovary and uterus. We note that several of their age and sex distributions were similar to the higher seroprevalence anogenital tumours, suggesting these factors did not determine the observed differences. Analysis of genital cancers by subtype was most remarkable for the relatively low HPV 16 VLP seroprevalence among the subset of cervical cancer patients with adenocarcinomas, a tumour which is most often associated with HPV 18 (IARC, 1995).

Tumours of the aerodigestive tract

HPV 16 VLP antibody prevalence rates were generally low in patients with non-genital cancers. The highest seroprevalence was in buccal cavity tumours. However, this result was less than half the rate detected in vulva or vagina. Patients with other cancers of the aerodigestive tract, including pancreas, rectum, colon and oesophagus had lower antibody prevalence rates. Analysis of aerodigestive cancers by histological subtype was not revealing.

Other cancers

Among the other cancers studied, the greatest seroprevalence was in patients with breast cancer. However, as above, this rate was less than half that detected in patients with cancers of the vulva or vagina, and less than a fifth of that detected in patients with penile or cervical cancer. Likewise, HPV 16 VLP antibody prevalence was relatively low in cancers of the bladder, melanomatous and non-melanomatous skin cancers, squamous and adenocarcinomas of the lung, and in kidney cancer. For completeness, we note that analysis of tumours by cancer subtype showed that HPV 16 VLP antibodies were present in two of four patients with lobular carcinoma of the breast, in the one patient with verrucous carcinoma of the skin, and in the one patient with Bowens' disease. However, the small numbers of these cases preclude further interpretation.

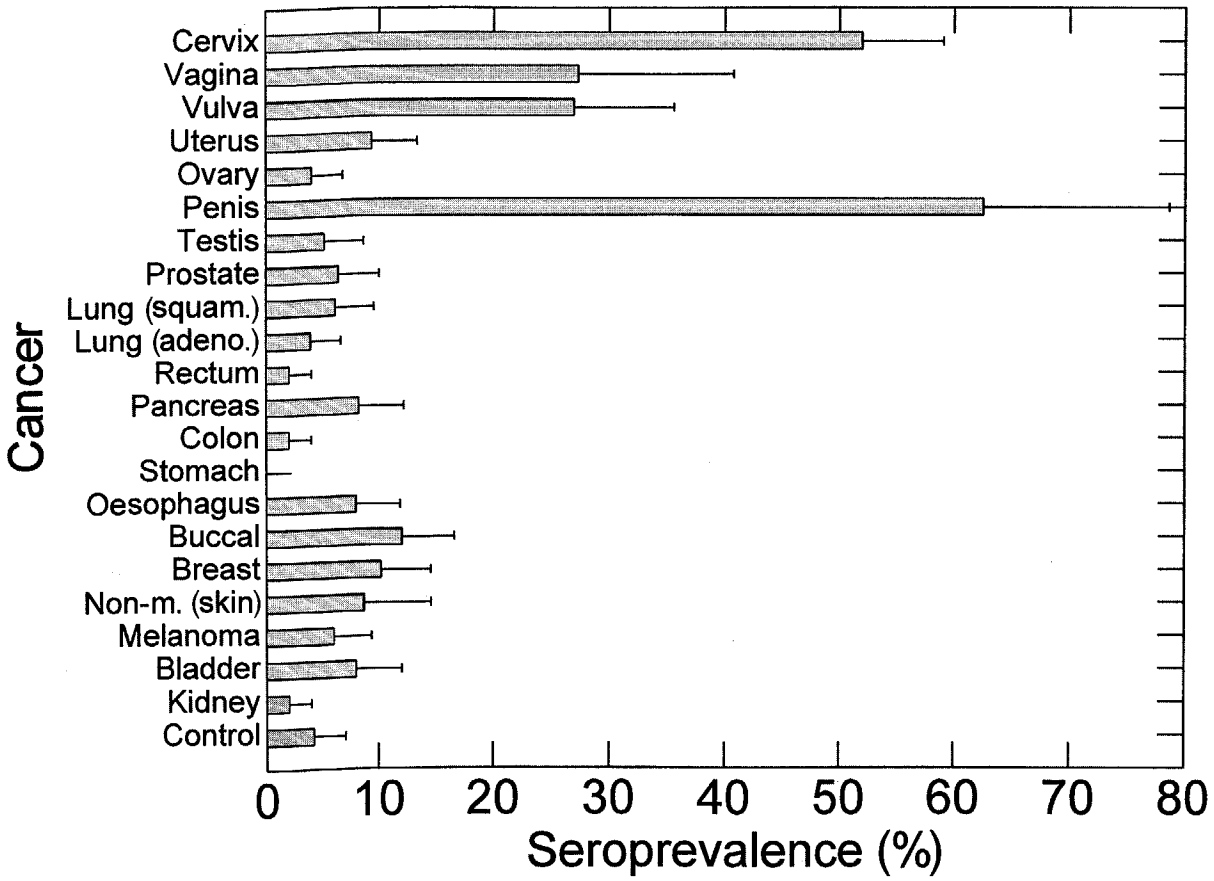

Figure 1. Seroprevalence of IgG to HPV 16 virus-like particles (VLPs) in cancer patients and in controls (non-cancer patients). The prevalence of antibodies in patients with each type of cancer is shown by the grey bars. The standard errors of these prevalence estimates are shown by the thin lines extending beyond each grey bar.

Discussion

Recent evidence suggests that HPV 16 leads to the development of cervical cancer, at least in part, by the production of viral proteins which interfere with normal cellular p53 and retinoblastoma tumour suppresser gene functions (IARC, 1995). These same mechanisms could also make HPV 16 tumorigenic in other epithelial tissues. In this study, we used a well characterized serological assay to measure exposure to HPV 16 infection in a large population of 905 patients with 21 different types of cancer that have varying degrees of evidence relating them to infection with HPV 16 (IARC, 1995; Franceschi *et al*, 1996).

The data are consistent with results of DNA studies suggesting that several tumours of the lower genital tract are possibly associated with HPV 16. Patients with cancers of the penis, cervix, vulva and vagina all had high HPV 16 VLP antibody prevalence rates. Specifically, seroprevalence was generally more than five times greater in patients with cancers of the penis (63%) or cervix (52%), and more than two times greater in patients with cancers of the vulva (27%) or vagina (27%) than in the non-cancer group (4%) or patients with other tumour types. It is reassuring in this context, to note that antibody prevalence in cervical cancer patients, the positive controls in this study, was similar to estimates of HPV 16 DNA prevalence in previous studies of cervical cancer specimens (Lorincz *et al*, 1992); likewise for penile, vulva and vaginal cancers (Ikenberg *et al*, 1990; Schneider *et al*, 1991; Maden *et al*, 1993; Malek *et al*, 1993; Gregoire *et al*, 1995; IARC, 1995; Trimble *et al*, 1996). Thus, both serological and DNA research strongly suggest an association of HPV 16 with these specific genital tumours.

In contrast, other genital cancers, including prostate (6%), testis (5%), ovary (4%) and uterus (9%) did not have high prevalence of antibodies against HPV 16 VLPs. The lack of association between prostate cancer and HPV 16 in this study, confirms the result of a recent investigation we conducted using multiple HPV DNA detection and serological methods (Strickler *et al*, 1998). Likewise, evidence of HPV DNA in cancers of the testis, ovary and uterus is sparse, and recent investigations have tended to be negative (Wong *et al*, 1993; IARC, 1995; Trottier *et al*, 1995; Czerwendka *et al*, 1996; Rajpert-De Meyts *et al*, 1996).

Neither did the data suggest a strong association of HPV 16 antibodies with any non-genital cancers. The generally low prevalence of HPV 16 antibodies in patients with aerodigestive cancers is particularly noteworthy, since there has been considerable recent debate regarding the purported association of these epithelial tumours with HPV 16 (Franceschi *et al*, 1996). The highest seroprevalence in an aerodigestive cancer was detected in patients with tumours of the buccal cavity (12%). However, this rate is considerably less than in patients with penile, cervical, vulva or vaginal cancers and contrasts with the high prevalence of HPV 16 reported in some early DNA hybridisation studies of buccal cancer. Among the other cancers studied, patients with tumours of the breast (10%) had the highest seroprevalence. However, this is again a low rate relative to results in lower genital cancers, and the DNA literature does not strongly support an association of breast cancer with HPV infection (Bratthauer *et al*, 1992; IARC, 1995). Overall, therefore, the data do not indicate a high frequency of infection with HPV 16 in patients with non-genital epithelial cancers in this population of mid-western USA patients.

The latter caveat is important because the epidemiology and presumably the aetiology of various epithelial cancers may be different in populations outside of the USA. In particular, an earlier serological study in Shaanxi, China and another in Finland found elevated HPV 16 VLP antibody prevalence in patients who developed oesophageal cancer (Dillner *et al*, 1995; Han *et al*, 1996) and in a study of penile cancer in Hunan, China, a mostly monogamous population, no HPV antibodies at all were found in either cases or controls (Wideroff *et al*, 1996). It is also important to note that, despite the large size of the current investigation, it was not designed to detect associations with HPV 16 that are limited to a small subset (or subtype) or a given cancer. For example, the literature concerning buccal cancer increasingly supports a role for HPV 16 in an uncertain minority of tumours (Steenbergen *et al*, 1995). Lastly, the study might have been insensitive to cancer associations in which HPV does not productively infect the tissue (since capsid antibodies would not be induced) or associations in which HPV types other than HPV 16 are involved. The latter is a minor concern, though, since HPV 16 was the predominant type found in essentially all non-genital cancers purported to be HPV-related (IARC, 1995; Franceschi *et al*, 1996).

In summary, this study supports the relation of several tumours of the lower genital tract with HPV 16 infection (essentially, the same ones for which the DNA evidence is strongest) and fails to verify controversial reports that HPV 16 may be associated with a high proportion of several types of non-anogenital cancers, although the possible role of HPV in a subset of these tumours cannot be discounted. We conclude that with improvement, serology could have a role in cancer screening programmes, and that therapies directed against HPV could play a role in future treatment strategies for anogenital tumours. Moreover, a substantial fraction of anogenital cancers might be preventable by HPV vaccines that are currently under development (Munoz *et al*, 1995; Schiller and Okum, 1996).

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